Exploring the Role of an Adipocyte-Specific Knockout of MHCII on Inflammatory-Driven Complications of Obesity in Female Pro-atherogenic Mice

An Undergraduate Thesis

Written and presented in partial fulfillment of the requirements for *Graduation with Research Distinction* in the College of Medicine at The Ohio State University

By: Dana Middendorf

Biomedical Science Major, The Ohio State University

2021

Honors Committee:

Dr. Willa Hsueh, MD, Research Advisor

Dr. David Bradley

Dr. Claudia Mosley, Honors Advisor

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Willa Hsueh for the opportunity to work in the lab, and for her mentorship and guidance these past four years. Working in a diabetes lab had been a dream of mine in the past, and I'm incredibly grateful for the support and opportunities I have been given to participate in various aspects of the research, as well as to carry out this project.

I would also like to thank Valerie Wright, who has been an amazing mentor and source of instruction throughout my time in the lab. Thank you, not only for the countless opportunities to assist on experiments, but also for your personal investment in my learning and help in every stage of this project, for your advice, and for caring for me as an individual. Thank you, Alan Smith, Joey Liu, and Alecia Blaszczak for all of the training you provided on various techniques and investing in me, and Dharti Shantaram for your support as I navigated writing up this thesis. I would also like to thank Dr. Bradley and Dr. Mosley for their assistance and patience in serving on my thesis committee, and extend a huge thanks to Gina Gebhardt, Olivia Pereira and Mia Shein for your help in the mouse handling, husbandry, and data collection throughout the process.

Finally, I would like to thank the College of Medicine, and specifically, the Biomedical Science major under the leadership of Steven Mousetes and Dr. John Gunn for their incredible support and guidance throughout my undergraduate career. And of course, my family – there's not much I could do without your love and support.

ABSTRACT

The adipocyte, in addition to functioning as an energy storage cell, is an important immune cell that links the innate and adaptive immune system. In obese humans, our laboratory has previously demonstrated that the major histocompatibility complex II (MHCII) pathway is one of the most differentially expressed between lean and obese adipocytes. Further work in high fat diet fed mouse models demonstrates that increased adipocyte MHCII expression resulted in pro-inflammatory T cell differentiation leading to insulin resistance and decreased glucose tolerance. In contrast, loss of adipocyte MHCII expression in aMHCII knockout male mice resulted in less inflammatory adipose tissue characterized by increased adipose tissue immunosuppressive regulatory T cells (Tregs) and prevented the development of insulin resistance and glucose intolerance induced by Western high fat diet (WD). Moreover, these mice had less atherosclerosis and less liver steatosis and inflammation, common complications of obesity. We, thus, aimed to understand the impact of the loss of aMHCII expression in female mice on these and other inflammatory complications.

We crossed aMHCII KO mice with proatherogenic low density lipoprotein receptor knockout (LDLR-/-) mice, aged the female animals for one year to accelerate atherosclerosis and hepatic injury, and fed them WD for twelve weeks (n=12 control, n=12 aMHCII KO). Glucose (GTT) and insulin (ITT) tolerance tests, body fat composition and fasting blood were collected prior to WD start and at study end. At the time of sacrifice, visceral adipose tissue was collected for T cell flow analysis and gene expression, liver

was collected for liver fat analysis and histology, and aortas were collected for atherosclerotic lesion area measurement.

Results showed that the female aMHCII KO mice experienced a significantly lower percent weight gain over 12 weeks than wildtype (control) littermate control mice, despite demonstrating no significant difference in overall percent body fat. The knockout mice additionally demonstrated improved glucose tolerance after 12 weeks of WD, compared to the control mice (AUC control = 23090.0 $\pm$ 520.9 vs. KO= 20442.3 $\pm$ 657.9, P=0.0045), although no difference was observed in insulin sensitivity. No difference was found in circulating plasma triglyceride or cholesterol levels; however, liver fat accumulation was significantly decreased in KO mice (control= 17.14 $\pm$ 0.93 %fat vs. KO= 13.89 $\pm$ 1.04 %fat, P=0.039). Finally, no difference was found in atherosclerosis % lesion area of the aorta. These results support that the adipocyte knockout of MHCII leads to improvement in the metabolic and hepatic phenotype in female mice after 12 weeks on WD. However, as the females demonstrated some variation from the effects seen in male mice, further investigation into the cause of these differences is warranted.

BACKGROUND AND SIGNIFICANCE

I. Adipocytes and obesity:

Obesity is a growing epidemic in the United States and worldwide, with over 75% of the U.S. adult population currently obese or overweight (Flegal, K.M., et al, 2012; Deng, et al, 2016). As the prevalence of obesity increases, so does the burden of its comorbidities, including type 2 diabetes mellitus, heart disease, metabolic syndrome, and an increased risk of developing certain cancers (Segula, D., 2016). Recent estimates of healthcare costs related to obesity exceed $200 billion annually, and costs are continually growing (Reynolds, T.H., et al, 2019). Obesity is characterized by both an expansion of adipose tissue (AT) and the development of a critical inflammatory state, which mediates many of these systemic complications.

Adipocytes are the cells which comprise the bulk of AT. They function as significant long-term energy storage and endocrine cells and have additionally been found to act as an important immune cell that links the innate and adaptive immune system (Deng, T., et al, 2013). In an environment of caloric excess, adipocytes are uniquely poised to respond by storing and releasing excess energy, and by secreting signals that initiate adipose inflammation. They also express major histocompatibility complex II (MHCII) molecules, which are cell-surface proteins responsible for presenting antigens to CD4+, or helper T cells and thus, regulating immune cell behavior (Berry, D.C, 2013).

Our laboratory has spent considerable time working to characterize the role of MHCII expression in AT inflammation and inflammatory-driven complications. In obese humans, we have shown that the MHCII pathway is one of the most differentially expressed

between lean and obese adipocytes (Deng, T., et al, 2013). MHCII acts as a critical determinant of an obesity-induced adipose T cell subset switch [3]. As MHCII expression is increased in adipocytes, a resulting change is seen in adipose resident T cells to a more pro-inflammatory state, characterized by recruitment of more pro-inflammatory interferon gamma producing T helper 1 (Th1) cells and fewer anti-inflammatory regulatory T (T reg) cells (Deng, T., et al, 2013) in humans. Together, these changes in adipose-resident T cell quantity and polarization in obesity lead to an increase in inflammation in the tissue and act as key determinants of systemic insulin action.

In high fat diet fed mouse models, we have additionally demonstrated that increased adipocyte MHCII expression results in pro-inflammatory T cell differentiation (Deng, T., et al, 2017). This switch in subsets of CD4+ T cells in the AT led to insulin resistance and decreased glucose tolerance. In contrast, loss of adipocyte MHCII expression in aMHCII knockout male mice resulted in less inflammatory adipose tissue, characterized by increased adipose tissue immunosuppressive Tregs. The loss of MHCII additionally prevented the development of insulin resistance and glucose intolerance induced by high fat, high cholesterol Western Diet (WD). Moreover, these mice experienced an improvement over controls in the development of fewer inflammatory complications (Deng, T., et al, 2017).

II. Inflammatory-driven complications

The inflammatory state within AT also induces multiple systemic complications, which affect over 180 million adults in the United States (American Liver Foundation, 2017; United States Census Bureau, 2019). Insulin resistance, which is a major

pathophysiologic change associated with type 2 diabetes, and metabolic syndrome has been linked with these pro-inflammatory T cell changes (Flegal, K.M., et al., 2007). Additionally, AT inflammation has been shown to play a role in the development of atherosclerotic plaques and non-alcoholic fatty liver disease (Blaszczak, A., et. al., 2019).

Atherosclerosis is characterized by an accumulation of lipids in the subendothelial space between the endothelium and smooth muscle tissues in the arteries, which increases the risk of developing coronary artery disease, heart attack, or heart failure (National Heart, Lung, and Blood Institute, 2019). Atherosclerotic plaques form as a result of long-term, non-resolving inflammation. It is characterized by a similar CD4+ T cell subset switch to an accumulation of pro-inflammatory Th1 cells due to their role in oxidative modification of lipids (Blaszczak, A., et. al., 2019). We have found that a repletion of anti-inflammatory T regs inhibits plaque progression in atherosclerosis-prone mice, confirming their role in attenuating inflammation (Deng, T., et. al. 2013). More recent studies have continued to characterize the effect of AT inflammation and immune-cell changes in atherosclerosis lesion size and complexity in the aorta (Blaszczak, A., et. al., 2019).

Another major complication associated with inflammation and obesity is non-alcoholic fatty liver disease (NAFLD). NAFLD is defined as an accumulation of fat in the liver, and is the most common liver disease worldwide, affecting nearly all individuals with obesity and insulin resistance (American Liver Foundation, 2017). Proinflammatory cytokines similar to those secreted by the adipocyte in obesity are also upregulated in the liver and combined with the accumulation of fatty acids, these conditions activate an inflammatory cascade (Blaszczak, A., et. al., 2019).

Understanding the nature of inflammation, specifically the pathways directed by MHCII molecules in the adipose tissue, is therefore vital to developing better therapies to combat these systemic inflammatory-driven complications. Current treatment options are limited to managing the comorbidities through diet and exercise, and if necessary, through management of insulin resistance, hypertension and hypertriglyceridemia (Blaszczak, A., et. al., 2019). There is currently no effective treatment for the management of NAFLD, which contributes significantly to morbidity and mortality related to obesity. Similarly, despite therapies such as statins or LDL reduction, atherosclerosis continues to pose a significant risk to obese humans, as progression overtime can lead to the development of coronary artery disease (Blaszczak, A., et. al., 2019). The link between AT accumulation and inflammation and these complications has recently been determined, and thus, the emerging potential for immunologic-based treatment options could be a promising route to decrease the impact of these comorbidities in the treatment of obese individuals in the future.

III. Previous findings and project aim

In a novel study, the Hsueh lab generated a mouse model with adipocyte-specific MHCII deficiency (aMHCII -/-) and used male aMHCII -/- and wild-type (control) mice to explore the role of MHCII upregulation in CD4+ adipose-resident T cell (ART) changes, adipose inflammation, and systemic insulin resistance (IR) (Deng, T., et al., 2013). It was found that mice with the knockout were protected from diet-induced IR due to the role of MHCII expression in adipocyte inflammation.

To study the role of MCHII expression on the other inflammatory-driven complications of fatty liver disease and atherosclerosis, we developed a pro-atherogenic LDLR-/- mouse model with an adipocyte knockout of MHCII (aMHCII -/-, LDLR -/-) (Blaszczak, A., et. al., 2019). The LDLR -/- mice contain a mutation that affects their low-density lipoprotein receptor (LDLR) (Khan, A., et al., 2018), leading to increased plasma levels of cholesterol on a normal chow diet, and more severe atherosclerotic lesions and liver fat accumulation on high-fat and high-cholesterol diets. It was found that the loss of adipocyte MHCII expression in these LDLR -/- aged mice reversed these effects and resulted in a nearly a 50% reduction in atherosclerosis and liver fat accumulation with no difference in weight or body fat accumulation between groups. These reductions in complications were associated with a maintenance of AT T regs, as opposed to the drop that typically occurs with high fat diet, highlighting a key immune function of the adipocyte in obesity through its regulation of AT T cells (Blaszczak, A., et. al., 2019).

Both of these aforementioned studies, however, were limited to using male mice. In order to more fully characterize the MHCII pathway, it is necessary to explore the mechanisms of MHCII expression in females.

Before beginning testing, it was understood that a sex-specific resistance to obesity has previously been found in female mice. A study by Jacobs et. al found that female LDLR -/- mice responded better to diets that promote atherogenesis and were more representative of human adults with cardiovascular disease than the male mice (Jacobs S.A.H., et al, 2019). The male models were more susceptible to the detrimental effects of high fat diet, resulting in increased adipocyte size, storage, and inflammation, as well as an increase in liver fat as compared to the females. In order to fully establish the role of

MHCII expression on inflammation and inflammatory-driven complications in our lab, we sought to perform testing in female cohorts of mice.

The objective of this study was to understand the impact of altering the immune cell populations in the AT through the inhibition of antigen presentation by MHCII on development of atherosclerosis and fatty liver disease in female aged pro-atherogenic mice. It was hypothesized that the female aged aMHCII -/- LDLR -/- (KO) mice on Western diet (WD) will exhibit improved glucose tolerance and insulin sensitivity and be better protected from diet-induced CD4+ ART changes, atherosclerosis, and fatty liver disease in comparison to the LDLR -/- control mice.

With a better understanding of how MHCII expression affects systemic inflammatory-driven complications, the lab hopes to move toward eventually finding potential therapies to prevent and alleviate these detrimental comorbidities of obesity.

METHODS

I. Mouse models

Previously generated aMHCII -/- mice (AdipoCre, H2Ab1 flox/ flox) were crossed with pro-atherogenic low-density lipoprotein receptor knockout (LDLR-/-) mice to generate litters of LDLR-/- (control) and aMHCII LDLR-/- (KO) animals. The knockout strategy of aMHCII consists of LoxP sites flanking exon1 of H2-Ab1 for its exclusion (Deng, T., et al, 2013). LDLR -/- mice were initially obtained from Jackson Laboratory.

After breeding the animals, PCR genotypic analysis was employed by TransnetYX to determine the genotype of the female control and KO mice. The mice were aged for one year on standard chow diet to accelerate atherosclerosis development and hepatic injury before starting a high fat, high cholesterol diet (Western Diet) at roughly 52 weeks of age. All female animals were group housed, kept on a 12-hour light dark cycle and are cared for according to animal use and care protocols approved by The Ohio State University Institutional Animal Care and Use Committee within pathogen-free animal facilities. Sample size for this project was n=12 control, n=12 aMHCII KO mice in total, spread across 3 separate cohorts of mice. The first cohort of n=5 and n=3 KO began baseline testing in March of 2018; the second of n=2 and n=3 KO began in April of 2018, and the third of n=5 and n=6 KO began in October of 2020. Average age at study start for all three cohorts were control: 53.3 $\pm$ 1.3 weeks vs KO: 51.7 $\pm$ 1.4 weeks, p=0.416.

At baseline, prior to diet change, animals are assessed for body weight, % body fat, insulin and glucose tolerance, and fasting blood draws, which is additionally repeated

at study end. Techniques and experimental strategy for these metabolic studies are discussed below.

II. Body Composition and Metabolic analyses

Body mass is measured to 0.1g for each animal at baseline, at the same time of day each week while the mice are on WD, and at sacrifice after the completion of the 12 weeks. The percent body fat measurements are taken with the EchoMRI machine for each animal at baseline and 12 weeks of diet. Percent fat total is calculated as a percentage of fat detected by the machine over total measured body weight.

Glucose tolerance and insulin sensitivity is assessed at baseline and at 12 weeks of WD through glucose tolerance tests (GTT) and insulin tolerance tests (ITT). For ITTs, chow is removed 6 hours prior to test start. The animals are fasted overnight, or 16 hours prior to GTTs. In both tests, baseline blood glucose measurements are collected with the Contour Next Easy meter and Contour Next blood glucose test strips using tail vein blood samples. Blood glucose values are additionally measured 15, 30, 45, 60, 90, and 120 minutes after intraperitoneal glucose injection (1g/ kg body weight), and 15, 30, 45, 60, and 90 minutes after intraperitoneal insulin injection (0.75U/ kg body weight). Normalized glucose values and area under the curve data is calculated using Microsoft Excel. A student paired-t test of raw measurements and normalized glucose values for the two groups of mice provides a measurement of significance of $p<0.05$. Additionally, consistent glucose measurements of a mouse that fall more or less than two standard deviations from the mean for the group were considered for exclusion of the individual mouse data

from the experiment. Outlying values could occur due to an issue in the injection of insulin or glucose, or due to poor general health of the animal.

For fasting blood draws, animals are fasted for 6 hours prior to a submandibular bleed at the right jaw. Blood is collected in EDTA tubes, transferred to ice and immediately processed within the lab for plasma isolation. The blood is first spun at 2,000 x g for 10 minutes, then after centrifugation, the upper plasma layer is transferred to a new tube and placed in the -20°C freezer until analysis by ELISA. For each sample, glucose, insulin, leptin, triglycerides and cholesterol is quantified in duplicate to ensure accurate measurements. Glucose levels are quantified using the Contour Next Easy meter with Contour Next blood glucose test strips and then the plasma samples are sent to the University of Cincinnati Medical Center Mouse Metabolic Phenotyping Center for insulin, cholesterol and triglyceride quantification. Aliquots of plasma are also sent to the University of Michigan ULAM In-Vivo Animal Core for quantification of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) liver enzymes by ELISA.

Circulating glucose and insulin levels are used to calculate Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) for the animals according to the formula: fasting insulin (μU/mL) x fasting glucose (mg/dL)/405. A lower HOMA-IR value corresponds with increased insulin sensitivity.

III. Aorta Dissection and Sudan IV Staining

To begin atherosclerosis analysis, the aortas are pulled from the sacrificed animals and flushed with 10ml of phosphate buffered saline (PBS) and then dissected en-bloc from the heart to the iliac bifurcation. External fat and connective tissues are removed

through cutting and pulling with dissection instruments, and then the cleaned aortas are placed in 10% phosphate buffered formalin for at least 24 hours before transferring to 70% ethanol. The full aorta tissues are then cut along the midline to open the aorta, and they are pinned to a silicone tray with 0.2mm insect pins.

Aortas are stained with Sudan IV solution (consisting of 0.5% Sudan IV wt/v, 35% ethanol/50% acetone) for 15-30 minutes at room temperature with continuous shaking. If necessary, 70% ethanol is used to de-stain Sudan IV particles from the aorta with continuous shaking to ensure a clear stain. Plaques appear pink, as the Sudan IV stains for lipids and triglycerides. Next, aortas are imaged using a dissecting microscope. Using ImageJ software, the total area of the aorta and lesions are each traced, then the measured % area of lesion coverage by atherosclerotic plaques is calculated for the arch portion and whole aorta. After all images are captured and analyzed, the aortas are washed in 80% ethanol until destained.

IV. Liver Fat Analysis

Liver fat accumulation is assessed in the mice using a portion of the liver isolated at the sacrifice of the animals. Livers are removed, and a portion of the right lobe is placed on ice until EchoMRI biopsy analysis. Data from the EchoMRI machine is used to calculate a fat % total of the liver using the formula: fat (g) / (fat + lean (g)) x 100%.

Paraffin-wax embedded liver samples are additionally stained with hematoxylin and eosin staining and sliced to create tissue slides. Tissue composition and cellular structure are visualized and photographed with the Echo Revolve Brightfield microscope.

V. Statistical Analysis

All data are presented as means ± SEM. Student t-tests were done for all comparisons with significance indicated at (*$p<.05$ and **$p<.01$).

RESULTS

I. Body Weight and Percent Body Fat

We found no differences in body weight or percent body fat between control and KO mice, however, when normalized to percent weight gain by mouse, the KO mice gained significantly less weight than control littermates. Average body weight at baseline showed no differences between control and KO mice (21.8 ± 0.4 g. vs 21.7 ± 0.3 g., p=0.939). Additionally, no difference was seen in average body weight at 12 weeks of WD between groups (30.0 ± 1.2 g. vs 27.8 ± 0.9 g., p=0.140). Percent weight gain over 12 weeks of WD was significantly lower for KO mice (37.7 ± 4.0% vs 27.6 ± 2.8%, p=0.049). (n=12 control, 12 KO mice). Percent body fat showed no differences at baseline (12.15 ± 1.09% vs 12.42 ± 2.27%, p=0.935; n= 2 control, 3 KO), or at 12 weeks (32.49 ± 2.11% vs 28.85 ± 2.63%, p=0.296; n= 7 control, 6 KO). Results are depicted in **Figure 1** below.

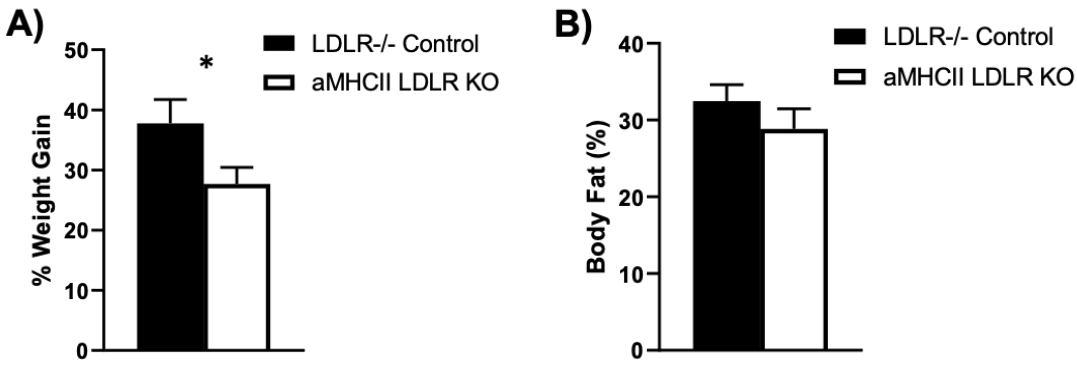

Figure 1: Percent Weight gain and body fat composition after 12 weeks WD. **A)** KO females gained significantly less weight after 12 weeks of WD, however, **B)** % body fat showed no differences. (n= 12 control, 12 KO).

II. Glucose Tolerance and Insulin Tolerance Testing (GTT and ITT)

In order to measure degree of insulin resistance and impaired glucose tolerance, baseline and 12-week GTTs and ITTs were conducted. Baseline date showed no significant differences between groups in glucose tolerance or insulin sensitivity. In performing baseline ITTs, the mice were given insulin doses of 0.50 U/ kg body weight, as opposed to the 0.75U/ kg body weight dose due to relatively low starting weights of the mice and the desire to avoid hypoglycemic events in the mice during baseline testing.

At 12 weeks of WD, repeated GTTs showed an improvement in glucose tolerance for the KO animals. The curve of blood glucose values over time showed a significant improvement at most time points, additionally, average area under the curve (AUC) calculations showed a dramatic improvement over the wild-type controls (23090.0 ± 520.9 vs 20442.3 ± 0657.9, $p=0.0045$). Results are shown in **Figure 2** below.

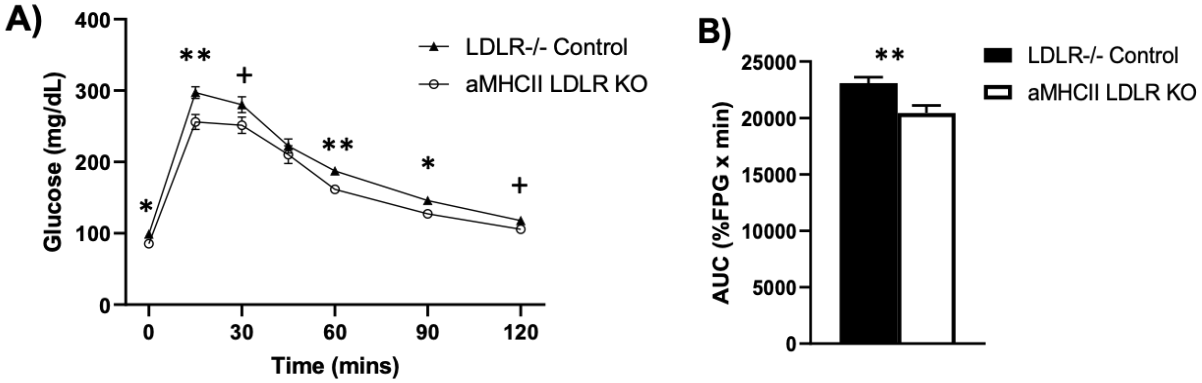

Figure 2: 12-week glucose tolerance test and average AUC data. **A)** KO mice demonstrate improved glucose tolerance in 12-week GTT curve of blood glucose values, with significant improvements at 0, 15, 30, 60, and 90 minute time points, and trending significance at 30 and 120 minutes. **B)** Area under the curve data also showed dramatic improvements. (n=12 control, 12 KO).

ITTs were additionally repeated at 12 weeks WD, however, at this time, the mice received the higher dose (0.75U/ kg body weight) of insulin, as they weighed significantly more at 12 weeks on WD than at baseline. Results showed no significant difference between control and KO groups, especially when glucose values were normalized to % initial curve and % initial average AUC (% initial AUC: 4872.9 ± 308 vs 4444.2 ± 193, p=0.251). n= 12 control, and 12 KO animals were tested in each metabolic test with no outlying data for exclusion. Results for the 12-week ITT are illustrated below in **Figure 3**.

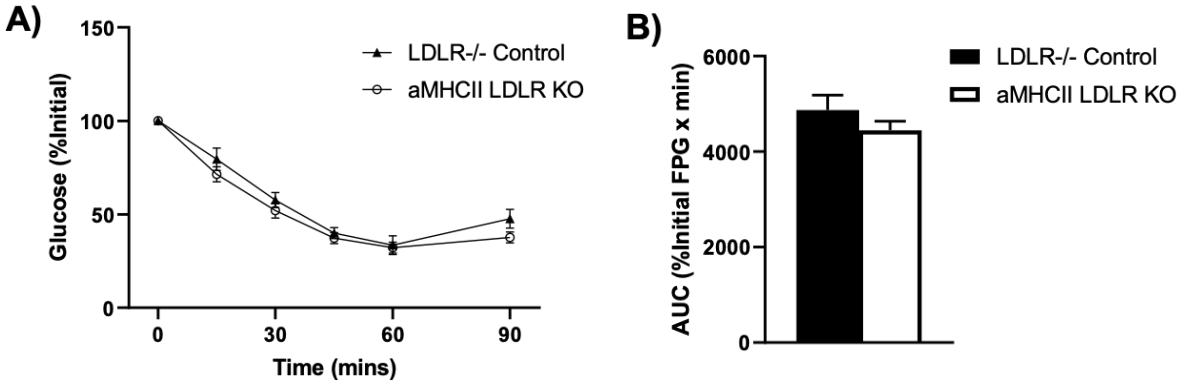

Figure 3: 12-week insulin tolerance test and average AUC data. **A)** No differences were observed in 12-week ITT curve of % initial blood glucose value at any time point. **B)** %initial area under the curve data also showed no difference in insulin sensitivity between groups. (n=12 control, 12 KO).

III. Plasma analysis

Baseline fasting blood draws and plasma analysis was performed for n=6 control, and 7 KO animals. No differences could be observed in circulating glucose, triglyceride, or cholesterol at study start. Due to a mix-up at the testing facility, insulin levels were not measured at baseline, however levels of circulating leptin were measured and additionally

showed no difference between groups. Without insulin data, HOMA-IR could not be calculated. Finally, samples were not sent for measurement of ALT or AST enzyme levels at baseline.

At 12 weeks of WD, fasting blood draws were performed on the same cohorts of animals, and the plasmas were analyzed for glucose, insulin, leptin, triglyceride, cholesterol, ALT and AST enzyme levels. No differences were observed in any of the measurements, including HOMA-IR calculation, as illustrated in **Figure 4** below.

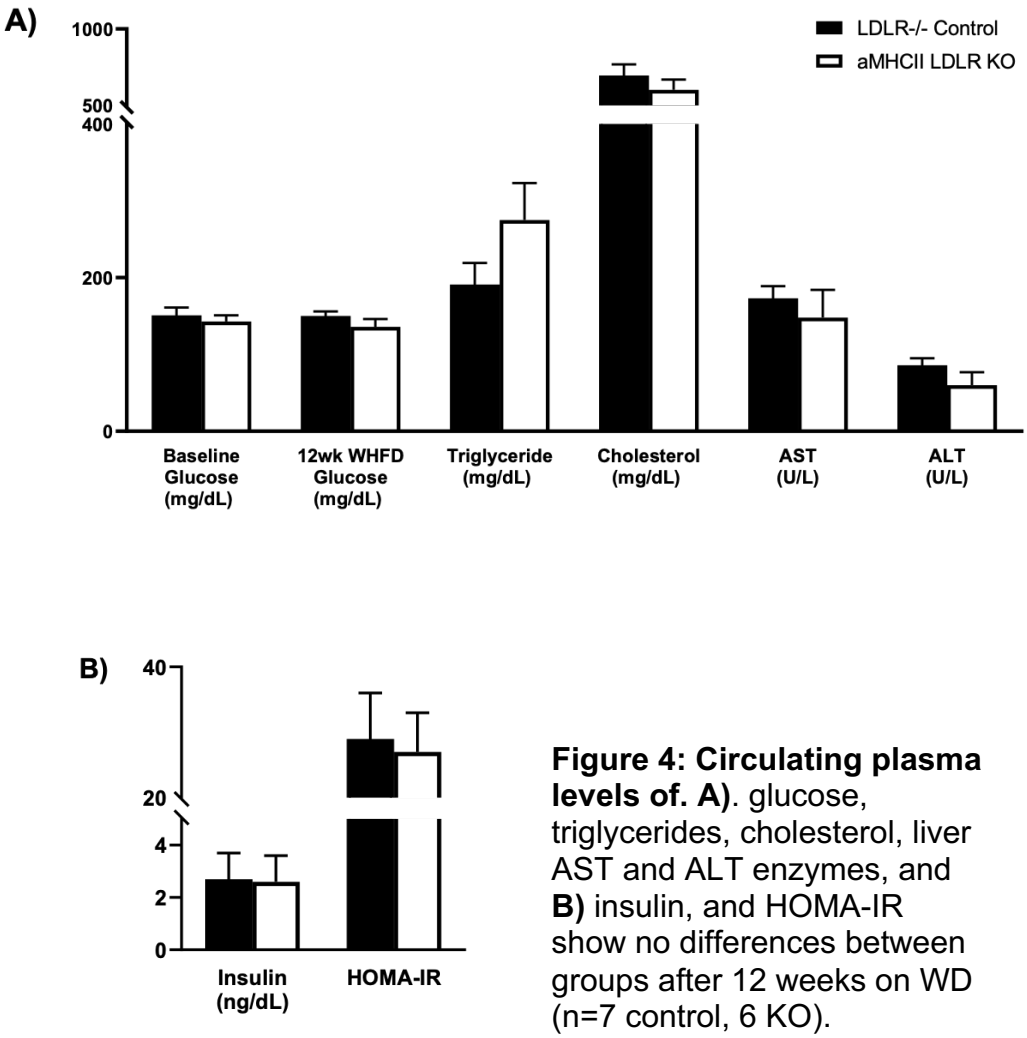

Figure 4: Circulating plasma levels of. A). glucose, triglycerides, cholesterol, liver AST and ALT enzymes, and **B)** insulin, and HOMA-IR show no differences between groups after 12 weeks on WD (n=7 control, 6 KO).

IV. Liver Fat Accumulation

EchoMRI tissue percent fat analysis of liver segments taken from female mice at sack showed that liver fat accumulation was significantly decreased in KO animals (17.1 ± 0.9% vs 13.9 ± 1.0%, p=0.0390; **Figure 5**). Representative H&E-stained slides of control and KO animals visually show smaller fat droplets in KO mice, as illustrated below.

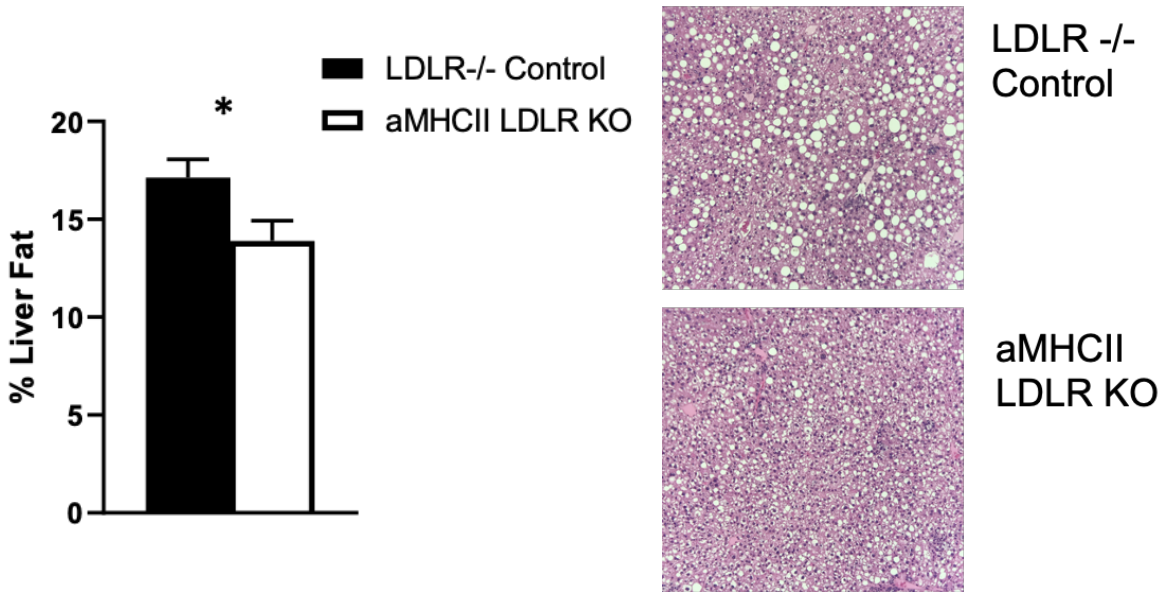

Figure 5: Liver fat accumulation after 12 weeks on WD. Liver fat accumulation was significantly decreased in KO females following 12 weeks WD (n=7 control, 6 KO).

V. Atherosclerotic Lesion Area

Analysis of atherosclerotic lesion area of the aorta showed no differences in % lesion coverage in the aortic arch (16.9 ± 3.7% vs 15.7 ± 4.0%, p=0.826), or the whole aorta (10.6 ± 1.9% vs 10.4 ± 2.8%, p=0.934). Representative images of Sudan IV-stained aortas additionally show no dramatic visual differences in lesion area coverage. Results of atherosclerosis analysis are depicted below in **Figure 6**.

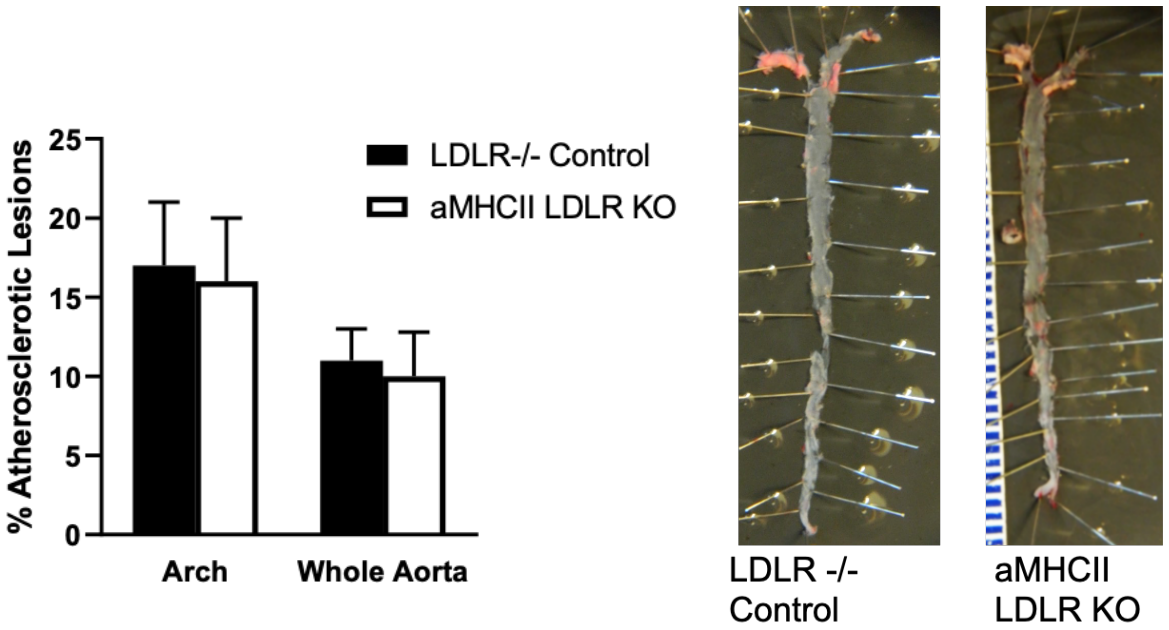

Figure 6: Atherosclerotic lesion area measurement of the dissected aortas. No significant difference was observed in atherosclerotic lesion area of the arch or whole aorta following 12 weeks WD (n=6 control, 6 KO).

DISCUSSION

Adipocyte MHCII expression in obesity is a critical determinant of systemic metabolic health. Investigation of the adipocyte MHCII pathway has repeatedly supported the role of the pathway in obese adipocytes in directly activating T cells and influencing adaptive immunity and adipose inflammation. Adaptive immunity, we've found, plays a significant role in the progression of obesity-induced adipose inflammation and insulin resistance, and more recent work has also illustrated the role of MHCII expression in inciting common inflammatory complications of atherosclerosis and liver fat accumulation (Deng, T., et al., 2013).

Historically, research into biological processes have largely overlooked the impact of sex. Numerous systematic reviews of animal research studies investigating biological processes have consistently found a significant overrepresentation of male mice in studies, and an even more significant lack of analysis of sex differences when both genders were employed (Beery A.K., 2010). The instance of sex differences is not limited to mice, however, as many known human disease processes vary widely by gender. These findings have led to a growing concern over the sex imbalances in research, as well as a concern for the poor generalizability of results in these research studies limited to males (Ingvorsen, C, et al., 2017).

The current investigation aimed to further explore whether inhibiting antigen presentation in the adipocytes, and thus, altering the immune cell subsets in the AT, would lead to a reduction in atherosclerosis and liver fat accumulation in female pro-atherogenic mice. Furthermore, as metabolic health of insulin and glucose tolerance has yet to be

assessed in female mice in the lab, it was important to characterize these effects of adipose inflammation in the females as well and assess any sex differences that emerged.

Our work in the females has confirmed that the loss of adipocyte MHCII leads to improvements in the metabolic and hepatic phenotype, similar to the male counterparts. Male pro-atherogenic mice with an adipocyte-specific knockout of MHCII saw improved metabolic health characterized by a dramatic decrease in atherosclerotic plaques and liver fat accumulation after 12 weeks on WD. Despite similar levels of weight gain and body fat accumulation between control and KO mice, the male mice displayed improvements in insulin resistance, as well as a nearly 50% reduction in both atherosclerosis development and liver fat accumulation (Blaszczak, A., et. al., 2019).

The male mice also consistently weighed more than the females, beginning at baseline testing and persisting throughout the testing period. However, the females and males had similar percent weight gain over the 12 weeks on WD, and additionally saw no differences in percent body fat at baseline or 12-week assessment. Body weight is a significant factor in impaired glucose tolerance and is strongly correlated with poor metabolic health, thus posing a significant confounding variable to the sex-driven differences (Ingvorsen, C, et al., 2017). Furthermore, studies have shown that total body fat, as opposed to percent composition or change might be the biggest driver of insulin resistance (Ingvorsen, C, et al., 2017). Other research has additionally shown that male LDLR -/- mice are more susceptible to developing larger and more inflamed adipocytes, which was in line with our findings (Reynolds, T.H., et al., 2019).

Regarding the effects of inflammation, past studies have also noted that female mice are generally more resistant to inflammation in response to diet-induced obesity (Pettersson U. S., et al., 2012). We found that the male mice trended or performed worse during GTT and ITT testing at baseline and 12 weeks than the females. However, within each gender, male KO mice saw improved insulin sensitivity to male controls, whereas female KO mice only saw an improvement in glucose sensitivity after 12 weeks on WD. It has been previously noted that male mice develop more pronounced systemic inflammation and insulin resistance than females, which have demonstrated a greater ability to expand the T reg population in the AT to counteract the pro-inflammatory cascade (Pettersson U. S., et al., 2012). In our testing, we found that circulating insulin levels were higher in control males than females, which has also been previously noted in studies of high fat diet-fed mice (Ingvorsen, C, et al., 2017).

Looking at the inflammatory complications of atherosclerosis development and liver fat accumulation, the females were generally affected by a lesser degree. Whereas female KO mice experienced an improvement in their hepatic phenotype, no difference was observed in the atherosclerotic lesion area between female groups. The male KO mice, in contrast, saw a nearly 50% reduction in both atherosclerosis and liver fat accumulation. Additionally, no differences were observed in circulating plasma triglyceride or cholesterol levels within each gender, however, males had significantly higher cholesterol levels within the KO mice than females, and increased triglycerides in both groups at baseline. Interestingly enough, however, when % liver fat data was compared across genders, the female KO animals actually had a greater average % liver fat than their male KO counterparts. In looking at plasma levels of ALT and AST liver

enzymes, no sex differences were seen, indicating no difference in these markers of liver damage. Liver fat accumulation is linked with obesity, insulin resistance, and elevated circulating triglycerides. Thus, further characterization of gene and cytokine expression in the liver may provide additional explanation to the higher % liver fat in female KO mice.

It is additionally important to discuss estrogen, which has been historically been a cause to exclude female mice from testing. At over a year of age, it is likely that our mice are beyond the known onset of menopause in mice, which occurs around 8 months (Reynolds, T.H., et al., 2019). Although estrogen can protect female mice from diet-induced obesity, our models were likely deficient in estrogen. The presence of estrogen can also decrease food consumption and promote energy expenditure to prevent obesity by creating a negative energy balance. One study found that removing the ovaries, and thus removing estrogen production from female ovariectomized mice no longer protected the females from obesity and restored their weight-gain pattern to that of the males (Stubbins, Renee E., et al., 2011). As we did not assess estrogen levels in our study, the potential for estrogen to influence our results is unknown and remains an important factor to consider in the future.

In summary, the current study continued to support the role of adipocyte MHCII expression in inciting AT inflammation, impaired glucose tolerance, an abnormal accumulation liver fat. As the female mice differed from the males in body mass, insulin sensitivity, glucose tolerance, and atherosclerosis lesions, our data supports the existence of sex differences in the development of inflammatory-driven complications and necessitates the consideration of both genders in metabolic studies moving forward. Better understanding the effect of MHCII expression on systemic inflammatory-driven

complications across genders paves a way to identifying potential therapies to prevent and alleviate these detrimental comorbidities of obesity in the future.

WORKS CITED

Akoum, S. E., et al. “Nature of Fatty Acids in High Fat Diets Differentially Delineates Obesity-Linked Metabolic Syndrome Components in Male and Female C57BL/6J Mice.” *Diabetology & Metabolic Syndrome*, BioMed Central, 14 Dec. 2011, dmsjournal.biomedcentral.com/articles/10.1186/1758-5996-3-34.

American Liver Foundation. What is fatty liver disease? 2017. https://liverfoundation.org/for-patients/about-the-liver/diseases-of-the-liver/nonalcoholic-steatohepatitis-information-center/nash-definition-prevalence/

Beery, A. K., and Irving Zucker. “Sex Bias in Neuroscience and Biomedical Research.” *Neuroscience & Biobehavioral Reviews*, Pergamon, 8 July 2010, www.sciencedirect.com/science/article/abs/pii/S0149763410001156?via%3Dihub

Berry, D.C., et al. The developmental origins of adipose tissue*. Development*. 2013; 140(19). 3939-49.

Blaszczak, A., et. al. Adipocyte-specific knockout of MHCII leads to an improvement in atherosclerosis and liver fat accumulation. (Unpublished data, 2019).

Deng, T., et al. “Obesity, Inflammation, and Cancer.” *Annual Review of Pathology*, U.S. National Library of Medicine, 2016, pubmed.ncbi.nlm.nih.gov/27193454/.

Deng, T., et al. (2017). "Adipocyte adaptive immunity mediates diet-induced adipose inflammation and insulin resistance by decreasing adipose Treg cells." Nature Comm 8: 15725.

Deng, T., et al., Class II Major Histocompatibility Complex Plays an Essential Role in Obesity-Induced Adipose Inflammation. *Cell metabolism*. 2013; 17. 411-22.

Flegal, K.M., et al. Cause-specific excess deaths associated with underweight, overweight, and obesity. *Jama*, 2007; 298(17). 2028-37.

Flegal, K.M., et al. Prevalence of obesity and trends in the distribution of body mass index among US adults, 1999-2010. *Jama*. 2012; 307(5). 491-497.

Ingvorsen, C, et al. "The Role of Sex and Body Weight on the Metabolic Effects of High-Fat Diet in C57BL/6N Mice." *Nature News*, Nature Publishing Group, 10 Apr. 2017, www.nature.com/articles/nutd20176.

Jacobs S.A.H., et al. Sex-Specific Differences in Fat Storage, Development of Non-Alcoholic Fatty Liver Disease and Brain Structure in Juvenile HFD-Induced Obese Ldlr-/-.Leiden Mice. *Nutrients*. 2019;11(8):1861. doi:10.3390/nu11081861

Khan, A., et al. Chapter 18 - In Vitro and In Vivo Animal Models: The Engineering Towards Understanding Human Diseases and Therapeutic Interventions. *ScienceDirect*, 2018; (1) 431-48.

National Heart, Lung, and Blood Institute. Atherosclerosis. *National Institutes of Health*. 2019. https://www.nhlbi.nih.gov/health-topics/atherosclerosis

Pettersson, U. S., et al. "Female Mice Are Protected against High-Fat Diet Induced Metabolic Syndrome and Increase the Regulatory T Cell Population in Adipose Tissue." *PLOS ONE*, Public Library of Science, 2012, journals.plos.org/plosone/article?id=10.1371%2Fjournal.pone.0046057.

Reynolds, T.H., et al. "The impact of age and sex on body composition and glucose sensitivity in C57BL/6J mice." Physiol Rep. 2019; 7(3). 13995.

Segula, D. “Complications of obesity in adults: a short review of the literature.” *Malawi medical journal : the journal of Medical Association of Malawi* vol. 26,1 (2014): 20-4.

Stubbins, R. E., et al. “Estrogen Modulates Abdominal Adiposity and Protects Female Mice from Obesity and Impaired Glucose Tolerance.” *European Journal of Nutrition*, Springer-Verlag, 1 Nov. 2011, link.springer.com/article/10.1007%2Fs00394-011-0266-4.

United States Census Bureau. United States QuickFacts. 2019. https://www.census.gov/quickfacts/fact/table/US/PST045218